

# Detection of Fungal DNA in Human Body Fluids and Tissues during a Multistate Outbreak of Fungal Meningitis and Other Infections

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*Exserohilum rostratum* was the major cause of an outbreak of fungal infections linked to injections of contaminated methylprednisolone acetate. Because almost 14,000 persons were exposed to product that was possibly contaminated with multiple fungal pathogens, there was unprecedented need for a rapid throughput diagnostic test that could detect both *E. rostratum* and other unusual agents of fungal infection. Here we report development of a novel PCR test that allowed for rapid and specific detection of fungal DNA in cerebrospinal fluid (CSF), other body fluids and tissues of infected individuals. The test relied on direct purification of free-circulating fungal DNA from fluids and subsequent PCR amplification and sequencing. Using this method, we detected *Exserohilum rostratum* DNA in 123 samples from 114 case-patients (28% of 413 case-patients for whom 627 samples were available), and *Cladosporium* DNA in one sample from one case-patient. PCR with novel *Exserohilum*-specific ITS-2 region primers detected 25 case-patients with samples that were negative using broad-range ITS primers. Compared to fungal culture, this molecular test was more sensitive: of 139 case-patients with an identical specimen tested by culture and PCR, *E. rostratum* was recovered in culture from 19 (14%), but detected by PCR in 41 (29%), showing a diagnostic sensitivity of 29% for PCR compared to 14% for culture in this patient group. The ability to rapidly confirm the etiologic role of *E. rostratum* in these infections provided an important contribution in the public health response to this outbreak.

In late September 2012, the Centers for Disease Control and Prevention (CDC) began to investigate an outbreak of fungal meningitis associated with epidural injections of contaminated methylprednisolone acetate solution purchased from a single compounding pharmacy (1–5). By 15 January 2013, 678 cases of fungal infections, including meningitis, spinal, paraspinal, or peripheral joint infections, had been reported from 19 states, and 44 people had died. Although *Aspergillus fumigatus* was isolated from the index patient (1), and several fungal species were identified in patients during the outbreak, the vast majority of laboratory-confirmed infections were caused by *Exserohilum rostratum*, which was detected in cerebrospinal fluid (CSF) and tissues of patients (3, 6).

*E. rostratum* (teleomorph *Setophafia rostrata*) is a dematiaceous (brown-black) filamentous fungus, which is common in the environment and occasionally causes opportunistic infections in humans (7–9). In healthy individuals, exposure to *E. rostratum* can cause allergic sinusitis or cutaneous and subcutaneous infections (7, 10, 11). Invasive fungal infections due to *E. rostratum* are rare and limited to persons with severely impaired immune systems (7, 10, 12). To our knowledge, no cases of fungal meningitis or encephalitis due to this fungus had been described prior to this outbreak. In addition, no genome or animal models had been developed to study *E. rostratum*, which also complicated the development and validation of a diagnostic test.

Detecting filamentous fungi in the human central nervous system is especially challenging because only a small number of these infections have been described (13). Recently, an outbreak of fungal meningitis caused by *A. fumigatus* was reported in post-tsunami Sri-Lanka and linked to the use of contaminated material during spinal anesthesia (14, 15). Among filamentous fungi, *A. fumigatus* is the most common cause of invasive fungal infections in clinical settings, so most of the available fungal detection meth-

ods target this organism and often rely on culture, antigen, and/or radiological findings (16–18). Although other filamentous fungi have been documented as causes of human meningitis and/or encephalitis, most of these infections have been diagnosed postmortem (19, 20). Furthermore, the sensitivity of fungus culture for detection of molds in CSF is very low (13, 18). A PCR method for detecting *A. fumigatus* in CSF has been proposed by Hummel et al.; however, its performance has not been well established since only a small number of cases have been evaluated (13, 21). A real-time PCR method for detecting *E. rostratum* DNA from fungal isolates has been recently described (22).

Here we describe the development of a method for purification of free-circulating fungal DNA from CSF and tissues and a novel PCR test for amplification of fungal DNA from sterile body fluids and sites. This PCR test relies on the amplification and sequencing of a portion of the ribosomal internal transcribed spacer region 2 (ITS2) using the ITS3/4 broad range fungal primer set (23, 24), as well as *Exserohilum*-specific primers developed for this investigation. Using this method, we confirmed the presence of *E. rostratum* DNA in samples from 28% (114/413) of case-patients and also detected the presence of *Cladosporium* DNA in one patient's specimen. Test results were available within 48 h after receipt of specimen and provided rapid confirmation of the presence of fungal DNA in patient samples.

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## MATERIALS AND METHODS

**Case definition and patient enrollment.** Clinical specimens were obtained from persons who were exposed to one of the implicated lots of preservative-free methylprednisolone acetate (MPA) produced by the New England Compounding Center after 21 May 2012 (4) and subsequently developed any of the following: meningitis of unknown etiology; posterior circulation stroke without a cardioembolic source and without documentation of a normal CSF profile; osteomyelitis, abscess, or other infection of unknown etiology in the spinal or paraspinal structures at or near the site of injection; or osteomyelitis or worsening inflammatory arthritis of a peripheral joint of unknown etiology diagnosed following joint injection (2, 5). Clinically diagnosed meningitis was defined as signs or symptoms of meningitis and a cerebrospinal fluid profile with pleocytosis ( $>5$  white cells per cubic millimeter), accounting for the presence of red cells. These case definitions have been previously described (5). A case-patient is defined as a patient who meets the case definition. In the early stage of the investigation, samples were also received from 136 patients who did not meet the case definition due to lack of CSF pleocytosis and also for other reasons, such as exposure to MPA not linked to the outbreak. These samples were tested, but the patients were later excluded from the formal investigation.

**Human subjects.** This investigation was considered an emergent public health response and therefore was not subject to review by CDC's Institutional Review Board.

**Specimens received.** This report includes specimens received between 2 October 2012 and 20 December 2012. Fluid and tissue samples from patients meeting the case definition or being evaluated for fungal meningitis or other infections were sent to CDC from state public health laboratories. Sterile body fluids included CSF, synovial fluids, and abscess aspirates. Tissues included spinal, paraspinal, and joint abscess tissues obtained by biopsy as well as a small number of brain and other central nervous system tissues from autopsy samples. In some cases, several specimen types, such as CSF, tissue, and synovial fluid, were obtained from the same patient. Multiple specimens were those collected on the same date or multiple specimens for which no dates of collection were provided. Serial specimens were those collected on different dates. Mixed specimens are different types of specimens, such as tissue and CSF, submitted either on the same day or on different days.

During the early stages of the outbreak, if serial samples were available, only the earliest unprocessed sample from that patient was tested. During later stages of the outbreak, all serial samples were tested when available. Early in the outbreak, some remnant samples that had not been kept frozen were sent for analysis. Otherwise, specimens were frozen as soon as possible after collection, were shipped on dry ice to the CDC, and were kept at  $-80^{\circ}\text{C}$  until testing.

**Sample processing.** At the CDC, all body fluids were thawed and equilibrated to room temperature, followed by centrifugation at  $18,000 \times g$  for 15 min; the resulting supernatant was collected and used for DNA purification. Several types of tissue samples were received: (i) large samples of fresh frozen intact tissue ( $\geq 0.5$  cm diameter), (ii) formalin-fixed paraffin-embedded (FFPE) tissue blocks, (iii) small samples of fresh frozen intact tissue ( $\leq 0.5$  cm in diameter), (iv) and tissue fragments in sterile saline from larger tissue biopsy specimens that had been minced in the submitting laboratory. Large samples of fresh frozen tissues (i) and fixed tissues (ii) were excluded from this investigation and are being reported separately (S. R. Lockhart et al., unpublished data; J. M. Ritter et al., unpublished data). (iii) Small samples of fresh frozen tissues were processed using the DNA extraction protocol for fresh tissues described below. (iv) Tubes containing tissue fragments sent in sterile saline were centrifuged and both pellets and supernatants were processed using the protocols for extracting DNA from fresh tissues and from body fluids described below.

**Purification of DNA from body fluids.** One milliliter of each supernatant obtained after centrifugation of body fluids or saline with suspended tissue fragments was collected for DNA purification using

QIAamp UltraSens virus kit (Qiagen, Valencia, CA) according to the manufacturer's instruction with the following modifications. (i) The volume of proteinase K was reduced to 4  $\mu\text{l}$  (one-fifth of the recommended amount) for sample digestion, and the lysis incubation (shaking at  $40^{\circ}\text{C}$ ) time was increased to 20 min. (ii) A low-speed centrifugation step ( $2,000 \times g$ ) was added after specimen lysis. (iii) Wash buffers were incubated on column for 5 min prior to centrifugation. (iv) Finally, the kit elution buffer (30  $\mu\text{l}$ ) was incubated on column for 5 min, and the elution step was performed twice. To monitor for extraneous fungal contamination, a negative control sample of PCR-grade water was included in each extraction procedure and processed in the same manner as for the patient specimens.

**Purification of DNA from fresh tissues.** Fresh tissues (small samples as well as pellets from the centrifugation of tubes containing tissue fragments in saline) were processed using a QIAamp DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions with the following modifications. (i) Specimens were resuspended in 180  $\mu\text{l}$  of Lyticase Z buffer (60 mM  $\text{Na}_2\text{HPO}_4$ , 40 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM KCl, 1 mM  $\text{MgSO}_4$ , 50 mM  $\beta$ -mercaptoethanol; pH 7) (25) and incubated with 20  $\mu\text{l}$  of proteinase K at  $55^{\circ}\text{C}$  until tissue was digested and for at least 1 h. (ii) After digestion with proteinase K, samples were incubated with 0.2 U of Lyticase (MP Biochemicals, Solon, OH)/ml for 1 h at  $33^{\circ}\text{C}$  with constant agitation. (iii) After digestion with Lyticase, each sample was mixed with one tube of BashingBeads from ZR Fungal/Bacterial DNA minikit (Zymo Research Corp., Irvine, CA) and vortexed at high speed for 5 min, after which the suspension was centrifuged at  $18,000 \times g$  for 15 min, and the supernatant was used for DNA purification. To monitor for extraneous fungal contamination, a negative control sample of PCR-grade water was included in each extraction procedure and processed in the same manner as patient specimens.

**Fungal culture.** Fungal culture was performed at the local clinical laboratory using local protocols and available results were reported to the CDC. In addition, available isolates were sent to CDC for confirmation. Culture results were not available from all specimens.

**PCR and sequencing.** Three primer pairs were used for nucleic acid amplification. (i) Broad-spectrum fungal primers ITS3 and ITS4 anneal within the conserved regions of 5.8S and 28S ribosomal DNA (rDNA) genes and amplify an  $\sim 350$ -bp fragment that includes the ITS2 region (ITS3, 5'-GCATCGATGAAGAACGCAGC; ITS4, 5'-TCCTCCGCTTAT TGATATGC) (24, 26). (ii) *Exserohilum*-specific primers were developed for this investigation and amplify the variable 230-bp region of ITS2 (Exs4F, 5'-GAAGAACGCAGCGAAATGCG; Exs4R, 5'-CCGAAAACCA GTAGGTCGCG). (iii) Positive control primers Beta2/Beta3 that amplify portions of the human  $\beta$ -globin gene (Beta2 [GH20], 5'-GAAGAGCCA AGGACAGGTAC; Beta3 [PC04], 5'-CAACTTCATCCACGTTCC) (27). Each sample was processed using all three PCR primer pairs.

PCRs were performed in 25- $\mu\text{l}$  volumes and contained 2.5  $\mu\text{l}$  of template DNA,  $1 \times$  PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 0.2 mM each of deoxynucleoside triphosphate, 1.6 mM  $\text{MgCl}_2$ , 0.2 pmol of each primer, and 0.6 U of *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN). The following PCR conditions were used: 4 min at  $94^{\circ}\text{C}$ , 40 cycles consisting of a denaturing step at  $94^{\circ}\text{C}$  for 1 min, an annealing step at  $52^{\circ}\text{C}$  for 1 min, and a polymerization step at  $72^{\circ}\text{C}$  for 1 min, followed by a single incubation step for 2 min at  $72^{\circ}\text{C}$ . PCR products were analyzed electrophoretically, and reactions that contained bands of the appropriate size were purified using ExoSAP (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Cycle sequencing reactions (20  $\mu\text{l}$ ) were prepared with 2  $\mu\text{l}$  of DNA template and 3.2 pmol of the same primers used for PCR, 2  $\mu\text{l}$  of BigDye Terminator v3.1, 2  $\mu\text{l}$  of sequencing buffer (Applied Biosystems, Inc.) according to the manufacturer's instructions. Extension products were purified using Centri-Sep plates (Princeton Separations, Inc.) and electrophoresed on a 3730 DNA analyzer (Applied Biosystems, Inc.).

**Interpretation of results.** DNA sequences from forward and reverse primers were assembled, manually edited, and compared to known sequences in NCBI GenBank using the BLAST algorithm. Sequences were

considered identical if they shared 100% identity with known sequences. If sequence data with a <20 PHRED quality score were obtained, the PCR was repeated, and the products were sequenced again (26). Even very faint PCR bands were sent for sequencing. If no readable sequence was obtained from the second attempt, the sample was deemed as “negative.” For case-patients with multiple or serial samples, the case-patient was deemed to have a “positive” result if at least one sample from this patient produced positive PCR results that were confirmed by sequencing.

**Determining limit of detection (LOD).** Genomic DNAs of *E. rostratum*, *Cladosporium cladosporioides*, and *Rhodotorula laryngis* were each combined 1:1 with human DNA and diluted to the various concentrations of fungal DNA—10 ng/ml, 1 ng/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml, and 100 fg/ml—using sterile water supplemented with 0.3 µg of bovine serum albumin/ml, which is the expected concentration of protein in normal human CSF. These solutions were subjected to DNA purification procedures using the QIAamp UltraSens virus kit, followed by PCR with ITS3/4 and *Exserohilum*-specific primers and DNA sequencing as described above. The lowest concentration of DNA for which PCR product and DNA sequence could be obtained was considered the LOD of this method (28).

**Determining diagnostic sensitivity and specificity.** Diagnostic sensitivity was defined as the fraction of samples correctly identified by PCR compared to isolation by culture. Because we did not have culture results for all samples and all patients, we estimated sensitivity of the PCR test by comparing PCR and culture results for a collection of 139 specimens, each of which was from a unique patient and each of which had a single specimen collection date for both culture and PCR results. We assumed that no more than a single lumbar puncture (LP) or biopsy procedure was performed on any given patient on the same day, and therefore all specimens submitted for culture and PCR on the same day from that patient originated from the same LP or biopsy sample. Since all of these patients met our case definition, we assumed that all patients had disease. Sensitivity was defined as the number of true positives/(the number of true positives + the number of false negatives). Diagnostic specificity was defined as the fraction of false-positive samples identified by PCR compared to culture. Because we did not have a patient population without disease, we determined specificity by using PCR results for 136 specimens obtained from patients who later were deemed not to meet the case-patient definition as described above.

## RESULTS

**Results of molecular detection.** We tested 627 samples from 413 case-patients who met the case definition for the outbreak, i.e., had a confirmed or probable case of meningitis or other infection. A total of 124 samples (20% of 627 samples) collected from 115 case-patients (28% of 413 case-patients) were found positive for fungal DNA by PCR and sequencing (Table 1). For 96 of these samples (77% of 124 positive samples) from 90 patients, PCR products of the appropriate size were observed with all three PCR primer sets. However, for 25 case-patients with 27 samples (22% of 124 positive samples) that were negative with the broad-range ITS primers, PCR products were obtained only when using *Exserohilum*-specific primers. Therefore, using *Exserohilum*-specific primers we were able to confirm presence of fungal DNA in 25 additional case-patients.

All positive PCR samples were confirmed by DNA sequencing, and with one exception, all were identified as *E. rostratum*. One CSF sample yielded product with the ITS3/4 primers but not *Exserohilum* primers, and this was identified as *Cladosporium* sp. after DNA sequencing and comparative analysis. All other sequences obtained with ITS3/4 or *Exserohilum*-specific primers shared 100% nucleotide identity with the ITS2 sequences of *E. rostratum* HE664064 compared to sequences in GenBank (9). Fur-

**TABLE 1** Results of PCR and DNA sequencing presented by patient and by sample

Results for:	No. tested	No. positive (%) <sup>c</sup>
All case patients <sup>a</sup>	413	115 (28)
CSF	345	83 (24)
Synovial fluids	15	3 (20)
Abscess aspirates	6	2 (33)
Tissues	71	27 (38)
All samples	627	124 (20)
CSF	506	91 (18)
Synovial fluids	18	3 (17)
Abscess aspirates	7	2 (29)
Tissues <sup>b</sup>	96	28 (29)

<sup>a</sup> Each case-patient is counted once. Note that some case-patients had more than one specimen type.

<sup>b</sup> FFPE samples are excluded.

<sup>c</sup> These values include 114 case-patients with *E. rostratum* and 1 case-patient with *Cladosporium* sp.

thermore, DNA alignment of all ITS2 sequences of *E. rostratum* from this outbreak indicated that all were indistinguishable at this locus.

During the initial assay validation step, 271 samples were subjected to a second round of PCR sequencing, and comparative analysis by a second CDC investigator yielded identical results. Early in the investigation, we also attempted to detect fungal DNA from CSF pellets using a bead-beater to break open putative fungal cells but were unable to obtain any positive results.

Control PCRs with human beta-globin primers were positive for all tissue and abscess samples and ca. 90% of fluid samples. The remaining 10% of samples, from which we were unable to amplify human beta-globin, were CSF and synovial fluids obtained from 136 patients who were later deemed not to meet the case definition and contained no measurable white blood cells. We hypothesized that rather than being contaminated by PCR inhibitors, these samples were devoid of human DNA. We added external control DNA to eight of these samples and observed amplification of the appropriate PCR products, which confirmed our hypothesis that these samples did not contain PCR inhibitors (data not shown).

**Performance in body fluids.** We tested 506 CSF samples from 345 case-patients and confirmed presence of *E. rostratum* DNA in CSF from 82 case-patients (24% of 345 case patients with CSF samples) and *Cladosporium* DNA in CSF from one case-patient (Table 1). In addition, we tested 18 synovial fluids and seven abscess aspirates from 21 case-patients. As shown in Table 1, three synovial fluids from three patients and two abscess aspirates from two patients were positive for *Exserohilum* DNA. In addition, we tested other miscellaneous samples such as serum, syringe, and swab wash samples, but none were positive (data not shown).

**Performance in tissue samples.** We tested 96 tissue samples obtained from 71 patients and found that 28 samples (29% of 96 samples) from 27 case-patients (38% of 71 case-patients) were positive for *Exserohilum* DNA. Most of the tissue samples were shipped to CDC as tissue fragments submerged in sterile saline, and when available, we extracted DNA from both tissue fragments and saline. Extracting DNA from the saline supernatant was more likely to generate positive results compared to direct extraction



TABLE 2 Comparison of PCR results among serially collected CSF samples (*n* = 73 case-patients)

Sample type	No. tested
Serial samples have the same results	52
All positive	5
All negative	47
Serial samples have different results	21
First sample positive, then negative	16
First sample negative, then positive	5

from the tissue pellets: 23 saline samples (82% of 28 positive tissue samples) and only 5 of the corresponding tissue samples (18% of 28 positive tissue samples) were positive for *Exserohilum* DNA.

**Testing multiple and serial samples.** Two to nine multiple and/or serial samples were obtained from 108 case-patients (a median three samples/patient), and concordant PCR results were obtained for 74 of these case-patients: for 68, all multiple/serial samples were negative, and for 6, all multiple/serial samples were positive. For the remaining 34 case-patients, both positive and negative results were obtained when multiple or serial samples were tested.

The largest group of serial samples was CSF. Specifically, 197 serially collected CSF samples were received from 73 case-patients (range, 2 to 9 samples collected from 2 to 35 days apart). Concordant PCR results were obtained for 52 of these case-patients (71% of 73 case-patients). For 47 case-patients, all samples were PCR negative, and for 5 case-patients, all samples were positive for *Exserohilum* DNA (Table 2). For 21 case-patients (29%), PCR results obtained from serially collected samples displayed discordant results. In five of these patients, samples collected early in the course of infection were negative, while subsequent samples were positive. For the remaining 16 case-patients (76% of 21 case-patients with the discordant results), the earliest collected sample was positive, while subsequent samples were negative (Table 2).

**LOD.** As shown in Fig. 1, the limit of detection (LOD) of *E. rostratum* DNA by PCR with both broad-spectrum and specific primer pairs was 1 pg/ml. If we assume that the genome of *E. rostratum* is similar in size to the 43 Mb genome of a sister species, *E. turcicum* (29), our method can detect the presence of 23 ge-

TABLE 3 Comparison between PCR and culture results (*n* = 139 case-patients)

Results categorized by test performed	No. positive (%)
PCR positive only	27 (19)
Culture positive only	5 (4)
Positive by both PCR and culture	14 (10)
All PCR positive	41 (29)
All culture positive	19 (14)
Positive by either method	46 (33)

nomes of *E. rostratum* in 1 ml of CSF. Because other fungi were detected in contaminated vials of methylprednisolone associated with this outbreak (4, 30) and/or isolated from CSF of infected patients, we also tested the LOD of our broad-spectrum PCR using ITS3/4 primers with DNA of *C. cladosporioides* and *R. laryngis* and found it was <100 fg/ml (Fig. 1C and D), indicating that these are also detected at 10 genomes or less.

**Diagnostic sensitivity and specificity.** Same-day collected specimens from 139 case-patients were tested by both PCR and culture to assess diagnostic sensitivity of the PCR method (Table 3). We obtained 41 positive PCR and 19 positive cultures in this group. For 5 case-patients (4% of 139 case-patients) culture was the only positive result, and for 27 (19% of 139 case-patients) PCR was the only positive result. For 14 patients (10% of 139), positive results were demonstrated in both PCR and culture. Overall, the PCR method was more likely than culture to detect positive cases; however, using both methods we could detect 46 (33% of 139) case-patients. The sensitivity of PCR in this population was 29%, and the sensitivity of culture was 14%. Using PCR results for 136 specimens obtained from patients who later were deemed not to meet the case definition, no false positives were detected and the specificity was 100%.

DISCUSSION

Here we describe a method for direct detection of fungal DNA in body fluids and tissues using targeted PCR and DNA sequencing. Using this method we were able to confirm the presence of fungal DNA in 28% of 413 case-patients with available specimens, who were part of the outbreak of fungal meningitis and other infections. Comparative analysis of DNA sequences confirmed the presence of *E. rostratum* in 114 case-patients, and DNA of *Cladosporium* sp. was detected in one case-patient. The clinical significance of detecting *Cladosporium* DNA in patients remains to be determined; however, the presence of *E. rostratum* DNA in patient samples can be correlated with fungal infection. Although the majority of positive samples were CSF, 3 synovial fluids, 2 abscess aspirates, and 28 tissue samples were found positive for *E. rostratum* DNA (Table 1).

As shown in Table 1, we detected fungal DNA in 20% of submitted samples but from 28% of patients meeting the presumptive case definition. This apparent discrepancy between the number of positive samples and case-patients in the investigation was not surprising, since in 108 cases up to 9 serial samples from a single patient were submitted, and in only 6 of these patients were all samples consistently positive. Although we do not have treatment data for all patients, we suspect that many samples might have been collected following the initiation of antifungal therapy, which may decrease the fungal bur-

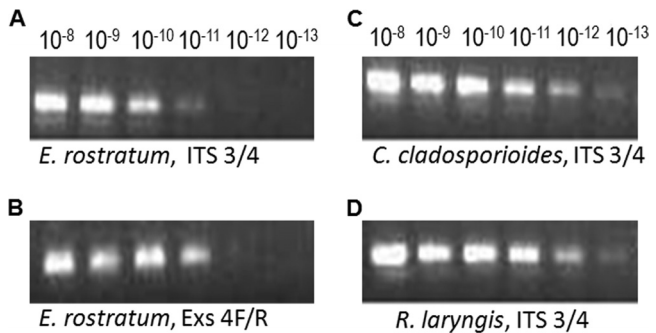


FIG 1 LOD of PCR detection. Serial dilutions of DNA of *E. rostratum* (A and B), *C. cladosporioides* (C), and *R. laryngis* (D) were subjected to DNA purification, PCR, and sequencing. Panels A, C, and D show results of the agarose gel with PCR products obtained using ITS3/4 primers, and panel B shows PCR results obtained using *Exserohilum*-specific primers. The numbers on the top of the gel depict concentrations of genomic DNA in grams per milliliter.

den in the central nervous system and therefore reduce the sensitivity of PCR (31).

The most challenging aspect of detecting filamentous fungi in body fluids and tissues by molecular methods is obtaining sufficient amounts of fungal DNA for testing. Until this outbreak, *A. fumigatus* was considered the most common filamentous agent of fungal meningitis, and several protocols have been developed for molecular detection of *A. fumigatus* DNA in serum and whole blood (32–34). These methods rely on capture of DNA from the pellet (presumed to contain intact fungal elements) following centrifugation. In the early phase of this investigation, the pellet and supernatant fractions of CSF or other body fluid samples were separated by centrifugation prior to purification, and both were processed for DNA purification. Although we were unable to obtain fungal DNA from the pellets, we successfully purified *E. rostratum* DNA from the supernatants. There are at least three nonexclusive explanations for our lack of success with DNA purification procedures from the pellet fraction of fluids. First, very few samples have been positive by culture, which suggests that *E. rostratum* hyphae do not circulate in sterile body fluids, and therefore fungal elements may not be present in the pellet fraction. Second, even if present, *E. rostratum* hyphae are heavily melanized and therefore may not be amenable to mechanical breakage or enzymatic digestion that are required by conventional purification protocols from hyphae and infected tissues. Third, the pellet fractions contain large quantities of human DNA, which competes with fungal DNA for binding sites on the purification columns and decreases yield of fungal DNA. To overcome these problems, we developed a different approach and successfully purified free circulating fungal DNA from the supernatant using a Qiagen DNA purification kit designed for isolating viral DNA from body fluids. Although the exact origin of this free circulating fungal DNA is unknown, we hypothesize that it is released after killing of fungal hyphae by immune cells.

We used a similar approach for purifying fungal DNA from tissues. Perhaps for the same reasons as discussed above, we had greater success extracting fungal DNA from the saline in which the tissue samples were transported than from the actual tissue fragments. By using saline extraction in combination with conventional DNA extraction from fresh tissues, we were able to detect *Exserohilum* DNA in 27 (38%) of the 71 case-patients for whom tissue samples were available. Extracting DNA from saline was more efficient compared to direct extraction from tissue fragments: only 5 specimens were found to be positive by using a direct tissue extraction method; however, 23 samples were found to be positive after extracting DNA from saline supernatants.

For PCR detection, we used two different sets of fungal primers: ITS3/4 broad-range fungal primers, and *Exserohilum*-specific primers that we developed specifically for this investigation. Both pairs of fungal primers target the ITS2 region of the rDNA operon, which has been successfully used for identifying fungal species and therefore is well-represented in sequence databases. The other advantage of using an ITS2 target for fungal detection is that this region is present in multiple copies in the genome and therefore is more likely to be detected compared to a single-copy gene. The results of the LOD experiments indicate that our assay can detect as little as 23 genomes of *E. rostratum* in 1 ml of body fluid. Although the LOD were the same for both sets of primers, *Exserohi-*

*lum*-specific primers were able to detect fungal DNA in an additional 25 case-patients whose samples were negative with ITS3/4 primers. This is most likely because *Exserohilum*-specific primers amplified a smaller region of ITS2 compared to ITS3/4 primers and therefore were more sensitive during amplification of partially degraded DNA as is frequently found in clinical samples. Because of the time constraints of the ongoing outbreak investigation, we did not test cross-reactivity of the *Exserohilum*-specific primers with other fungal pathogens and therefore did not rely on PCR for speciation. Instead, all PCR products were sequenced and each fungal pathogen was identified by comparing its DNA sequence with the known sequences in GenBank. The PCR and culture results were concordant in detecting *E. rostratum*, which was also cultured from unopened vials of MPA (4, 5). We also note that, although *Rhodotorula laryngis* was cultured from unopened vials of MPA (4), we never detected DNA of *R. laryngis* in any patient samples.

Comparison between results obtained by PCR and culture methods in a subset of case-patients with both results available from the same specimen demonstrated that molecular detection was more sensitive than culture in detecting fungus among patients, showing a sensitivity of 29% compared to a sensitivity of 14% for culture in this group (Table 3). Detection of fungal DNA in culture-negative case-patients was not surprising, since our DNA purification protocol was aimed at detection of free circulating fungal DNA and therefore can detect DNA from both living and dead fungal cells. However, our inability to detect fungal DNA in five samples that were positive by culture reflects the limitations in sensitivity of the PCR detection method, which might have been affected by sample quality and shipment speed. Specifically, four of these five samples were submitted in early October before shipment and storage recommendations for specimens were communicated to the clinical laboratories, and according to our records, these specimens were stored on site for 7 to 17 days prior to shipment to CDC for testing, which might have resulted in DNA degradation. Furthermore, although hospitals were advised to send samples frozen on dry ice, many samples arrived thawed and/or were stored at 4°C prior to shipment.

Despite these limitations, PCR testing was able to confirm the presence of fungal DNA in 28% of case-patients, and a combination of both PCR and culture demonstrated the presence of fungi in 33% of case-patients. Until experimental animal models of *E. rostratum* infection are developed, the true sensitivity of PCR and culture detection methods cannot be reliably estimated. A significant caveat of both PCR and culture detection methods is that negative results do not exclude infection and cannot be used to determine whether patients are free of infection. Overall, our results suggest that this method of PCR and sequencing can supplement culture of *E. rostratum* and detect additional cases of infection. This assay can also detect fungal pathogens other than *E. rostratum*. Molecular results can be generated within 48 h after specimen receipt compared to culture that takes 7 to 14 days for results; therefore, molecular detection can significantly accelerate diagnosis and treatment. Use of this assay played a significant role in the public health response to this outbreak by helping to confirm infections in patients rapidly, allowing for prevention of the potential for missed diagnoses in exposed patients and for better characterization of the outbreak.

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